

Applications of EcR Gene Switch Technology in Functional Genomics

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Genetic engineering of plants using transgenic technology is targeted to enhance agronomic performance or improved quality traits in a wide variety of plant species, and has become a fundamental tool for basic research in plant biotechnology. Constitutive promoters are presently the primary means used to express transgenes in plants. However, inducible gene regulation systems based on specific chemicals have many potential applications in agriculture and for enhancing the basic understanding of gene function. As a result, several gene switches have been developed. The ecdysone receptor gene switch is one of the best inducible gene regulation systems available, because the chemical, methoxyfenozide, required for its regulation is registered for field use. An EcR gene switch with a potential for use in large-scale field applications has been developed by adopting a two-hybrid format. In a two-hybrid switch format, the GAL4 DNA binding domain (GAL4 DBD) was fused to the ligand binding domain (LBD) of the *Choristoneura fumiferana* ecdysone receptor (CfEcR); and, the VP16 activation domain (VP16 AD) was fused to the LBD of *Locust migratoria* retinoid X receptor (LmRXR). The sensitivity of the CfEcR gene switch was improved from micromolar to nanomolar concentrations of ligand by using the CfEcR:LmRXR two-hybrid switch. In this report, we demonstrate the utility of CfEcR:LmRXR two-hybrid gene switch in functional genomics applications for regulating the expression of a *Superman*-like single zinc finger protein 11 (*ZFP11*) gene in both *Arabidopsis* and tobacco transgenic plants. Arch. Insect Biochem. Physiol. 65:164–179, 2007. © 2007 Wiley-Liss, Inc.

KEYWORDS: *Arabidopsis*; *Choristoneura fumiferana*; ecdysone receptor; *Locusta migratoria*; real-time PCR; Retinoid X receptor; tobacco; transgenic plants; Zinc Finger Protein 11; ecdysone agonist

INTRODUCTION

Overexpression of a gene is an effective method to investigate gene function in basic plant biology research as well as in biotechnology applications. The promoter often used to overexpress transgenes is the constitutive CaMV (cauliflower mosaic virus) 35S promoter (Odell et al. 1985). The CaMV 35S promoter is a strong promoter that is expressed in different developmental stages and tissues and in a wide range of plant species. However, the CaMV 35S promoter has some limitations; for example, it can not be used for those genes where a

loss-of-function and/or gain-of-function lead to lethality. Compared to constitutive promoters, a regulated promoter that allows conditional expression of a transgene makes it easier to get more precise and reliable results (Gatz, 1996; Gatz and Lenk, 1998; Zuo and Chua, 2000; Padidam, 2003; Palli, 2005). Inducible promoters greatly facilitate genetic analysis by providing a means by which to correlate a particular phenotype with the expression of a given gene. Inducible promoters also offer a way to study genes and gene products whose constitutive expression is otherwise detrimental or lethal to the plant (Schena et al., 1991). Attempts

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Contract grant sponsor: Kentucky Tobacco Research and Development Center (KTRDC), University of Kentucky.

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at solving some of the problems associated with constitutive expression of transgenes led to the development of gene regulation systems based on plant promoters that increase transgene transcription upon application of herbicide safeners (Jepson et al., 1994; De Veylder et al., 1997), plant hormones (Suehara et al., 1996), or heat shock (Severin and Schoffl, 1990). These systems supported a rapid induction of transgenes, but also induced the expression of endogenous plant genes that respond to these signals. Accordingly, these gene regulation systems have been used only to a limited extent.

The selective induction of gene expression is typically accomplished through the use of a promoter whose transcriptional activity is determined by the presence or absence of a specific inducer. In this context, several attempts have been made to develop chemically regulated gene expression systems (Gatz et al., 1992; Weinmann et al., 1994; Aoyama and Chua, 1997; Caddick et al., 1998; Martinez et al. 1999a,b; Bruce et al., 2000; Roslan et al., 2001; Zuo et al., 2001; Love et al., 2002; Padidam et al., 2003; Craft et al., 2005; Samalova et al., 2005). An ideal system should show a specific response to an inducer with no pleiotropic effects, should be able to provide repeated on and off responses, and should involve a non-toxic inducer that can be easily applied and that is effective at low concentrations (Tavva et al., 2006b). The ecdysone receptor (EcR) dependent expression system is one of the best gene regulation systems and responds to a registered insecticide for its regulation (Tavva et al. 2006b). Rohm and Haas Company have developed several non-steroidal agonists (biacylhydrazines) of EcR as insecticides for field use. These agonists bind EcR with nanomolar affinity, are non-phytotoxic, safe to many organisms, and, therefore, suitable for both laboratory and field applications (Dhadialla et al. 1998).

Recently, we described the construction of a two-hybrid *Choristoneura fumiferana* ecdysone receptor (CfEcR) gene switch and its induction characteristics in tobacco, corn, and soybean protoplasts and in transgenic *Arabidopsis* and tobacco plants (Tavva et al. 2006b). In this two-hybrid switch format, the

GAL4 DNA binding domain (GAL4 DBD) was fused to the ligand-binding domain (LBD) of the *Choristoneura fumiferana* ecdysone receptor (CfEcR), and the VP16 activation domain (VP16 AD) was fused to LBD of *Locust migratoria* retinoid X receptor (LmRXR) or *Homo sapiens* retinoid x receptor (HsRXR). Upon application of methoxyfenozide, the heterodimer of these two fusion proteins transactivates the luciferase reporter gene placed under the control of multiple copies of GAL4 response elements and a minimal 35S promoter. The sensitivity of the CfEcR gene switch was improved from micromolar to nanomolar concentrations of ligand by using the CfEcR:LmRXR two-hybrid combination and the reduction in the background expression levels in the absence of ligand was achieved by using a CfEcR+HsRXR two-hybrid combination. In this report, we tested the utility of the two-hybrid EcR gene switch (CfEcR+LmRXR) in functional genomics applications by demonstrating the regulation of the expression of a *SUPERMAN*-like single zinc finger protein 11 (*ZFP11*) gene isolated from *Arabidopsis thaliana* (*AtZFP11*).

A single zinc finger protein from *Arabidopsis thaliana* (*AtZFP11*) causes mortality and deformed phenotype when over-expressed under the control of a 35S promoter in both *Arabidopsis* and tobacco plants (Dinkins et al., 2003). We were unable to recover healthy transgenic *Arabidopsis* and tobacco plants expressing *AtZFP11* under the control of CaMV 35S promoter. As described in Dinkins et al. (2003), some 35S:*AtZFP11* transgenic tobacco plants were recovered, but all displayed abnormal phenotypes. Moreover, the seed collected from these transgenic tobacco plants failed to germinate on agar plates supplemented with kanamycin. Therefore, the *AtZFP11* gene is an ideal candidate for testing the application of the EcR gene switch in functional genomics. The CfEcR+LmRXR two-hybrid switch was used to make transgenic tobacco and *Arabidopsis* plants expressing *AtZFP11* gene. We monitored the expression of the zinc finger protein gene in transgenic plants prior to and after application of ligand and after withdrawal of the ligand using quantitative real-time reverse transcription PCR (qRT-PCR).

MATERIALS AND METHODS

DNA Manipulations

Binary vectors for stable transformation of *Arabidopsis* and tobacco were constructed in pCAMBIA 2300 vectors (CAMBIA, Canberra, Australia). To construct binary vectors for plant transformation, different promoter and terminator sequences were amplified by PCR, cloned into the pGEM®-T Easy vector (Promega Corporation, Madison, WI), sequenced to confirm their identity, and assembled into the pSL301 cloning vector (Invitrogen™, Life Technologies, Carlsbad, CA). The FMV (figwort mosaic virus) and MMV (mirabilis mosaic virus) promoter sequences were amplified from the pUCFMVFLt101 and pUM24 plasmids (Maiti et al., 1997; Dey and Maiti, 1999) as *Sall/XbaI* and *PstI/XbaI* fragments, respectively, and cloned into pSL301 vectors. The receptor fusion gene sequences (GAL4 DBD:CfEcR, VP16 AD:LmRXR) were restricted out as a *NheI/XbaI* fragment from pVP16 or pM vectors and cloned into the pSL301 vector downstream of either FMV or MMV promoter sequence. The ubiquitin 3 terminator (Ubi3 T) and *Agrobacterium tumefaciens* octopine synthase polyA (OCS T) sequences were amplified from the in-house plasmids as *XbaI/SpeI* and *HindIII/XbaI* fragments, respectively. For the target gene expression cassette, the -46 CaMV 35S minimal promoter was PCR amplified from the pKYLX80 vector together with the adjacent multiple cloning site and rbcS terminator sequence by using specifically designed primers flanked with *EcoRI* and *SpeI* restriction sites on the forward primer (*gaattc actagtGCAAGACCCTTCCTC*) and *Sall* and *ClaI* restriction sites on the reverse primer (*atcgat gtcgactGATGCATGTTGTCA*). The resulting PCR product (-46 35S-MCS-rbcST) was cloned into the pGEM®-T Easy vector (Promega Corporation) and the sequence was confirmed. The *EcoRI/ClaI* fragment of -46 35S-MCS-rbcST was restricted from pGEM-T Easy and cloned back into the pKYLX80 vector in the same restriction sites, so that the original full-length CaMV 35S promoter together with adjacent multiple cloning site and the rbcS terminator were replaced. Five copies of

the GAL4 response elements (5XGAL4 RE) were PCR amplified from the pFRLuc vector (Stratagene, La Jolla, CA) and cloned into the pGEM-T Easy vector to confirm the sequence. The *EcoRI/XbaI* fragment of 5X GAL4 RE was excised from pGEM-T Easy and cloned into *EcoRI/SpeI* sites upstream of the -46 35S minimal promoter in the modified pKYLX80 vector. The *Arabidopsis zinc finger protein 11* (*AtZFP11*) gene sequence was amplified from the cDNA prepared from the total RNA isolated from the *Arabidopsis* seedlings. Oligonucleotide primers were synthesized to include the restriction enzyme *XhoI* site adjacent to the ATG start codon and *SacI* downstream of the TAA stop codon for easy cloning in the forward and reverse primers, respectively (For: 5' ctc gag ATG AAG AGA ACA CAT TTG GCA 3'; Rev: 5' gag ctc TTA GAG GTA GCC TAG TCG AAG 3'). The resulting PCR product was cloned into the pGEM®-T Easy vector (Promega Corporation) and sequence verified. The *XhoI/SacI* (in lowercase letters in the primers above) *AtZFP11* fragment was excised and cloned into the *XhoI* and *SacI* site of the pK80-46 35S vector. The entire cassette (-46 35S:AtZFP11:rbcS T) was taken from the pK80-46 35S vector and cloned into the pCAMBIA 2300 plasmid (CAMBIA, Canberra, Australia) along with CfEcR and LmRXR expression cassettes for plant transformation. The different expression cassettes (FMV P:GAL4:CfEcR:Ubi T, MMV P: VP16:LmRXR:OCS T, -46 35S:AtZFP11:rbcS T) assembled in different cloning vectors were restricted and inserted into pCAMBIA2300. The resultant binary vector was designated as p2300CfE:LmR:AtZFP11 (Fig. 1a). The 35S:AtZFP11:rbcS T cassette taken from the pKYLX80 vector was cloned into pCAMBIA2300 to generate transgenic *Arabidopsis* plants that constitutively expressed the *AtZFP11* gene (Fig. 1b). The pCAMBIA2300 binary vector also has the kanamycin resistance gene expression cassette for transgenic plant selection (not shown in the Fig. 1).

Plant Tissue Culture

Arabidopsis thaliana (L.) Heynh. ecotype Columbia ER and *Nicotiana tabacum* L. cv. KY160 (University of Kentucky Tobacco Breeding Program)

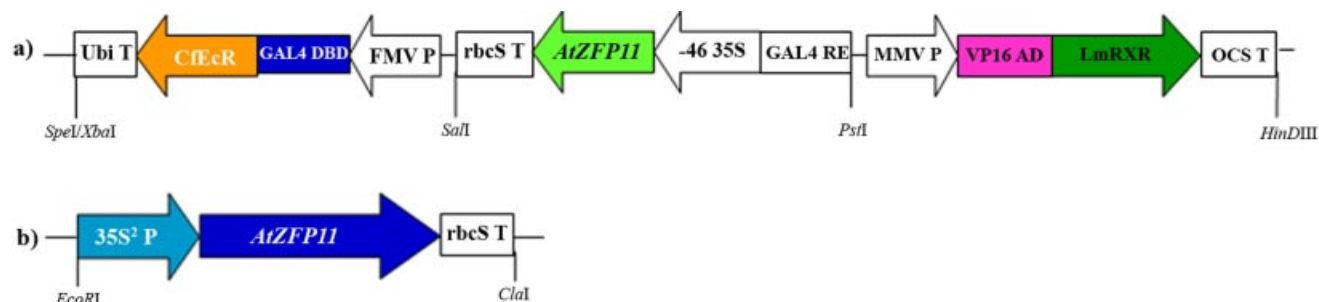


Fig. 1. Schematic representation of the binary vectors. **a:** p2300CfE:LmR:AtZFP11: T-DNA region of the pCAMBIA2300 binary vector showing the assembly of receptor (FMV P:GAL4 DBD:CfEcR:Ubi T; MMV P:VP16 AD:LmRXR:OCS T) and transgene (5X GAL4 RE:-46 35S: AtZFP11:rbcST) expression cassettes. The nptII expression cassette is not shown. **b:** p2300 35S:AtZFP11: T-DNA region of the pCAMBIA2300 binary vector showing the assembly of *AtZFP11* gene cloned under the control of a CaMV 35S promoter and rbcS terminator. 35S² P: a modified CaMV 35S promoter with duplicated enhancer region; *AtZFP11*:

Arabidopsis thaliana zinc finger protein 11 gene; rbcS T: Rubisco small subunit polyA sequence; FMV P: figwort mosaic virus promoter; GAL4 DBD: yeast GAL4 DNA-binding domain; CfEcR: *Choristoneura fumiferana* ecdysone receptor; Ubi T: ubiquitin 3 terminator; MMV P: mirabilis mosaic virus promoter; VP16 AD: *Herpes simplex* transcription activation domain; LmRXR: *Locusta migratoria* retinoid X receptor; OCS T: *Agrobacterium tumefaciens* octopine synthase polyA; -46 35S P: 35S minimal promoter.

were used for plant transformation experiments. The binary vectors constructed for plant transformation, p2300CfE:LmR:AtZFP11 and p2300:35S:AtZFP11 were mobilized into *Agrobacterium tumefaciens*, strain GV3850 by the freeze-thaw method. *Arabidopsis thaliana* plants were transformed using the whole plant-dip method (Clough and Bent, 1998). Transgenic *Arabidopsis* plants were selected by germinating seeds collected from the infiltrated plants on a medium containing 50 mg/l kanamycin. Resistant T₁ plants surviving on kanamycin-containing media were transferred to soil and then moved to a greenhouse for further analysis. Tobacco plants were transformed by employing standard leaf disc transformation protocols and media recipes (Schardl et al., 1987).

Dose-Response Study With T₂ Arabidopsis and T₁ Tobacco Plants

Seeds collected from the T₁ Arabidopsis plants and T₀ tobacco plants were plated on agar media containing appropriate amounts of kanamycin and different concentrations of methoxyfenozide (0, 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 nM). The seeds were allowed to germinate and grow on the

induction media for 20 days in the case of *Arabidopsis* and 4 weeks in the case of tobacco at 25°C, 16 h light/8 h dark.

Quantitative Real-Time PCR (qRT-PCR)

The expression levels of the *AtZFP11* gene in transgenic tobacco and *Arabidopsis* plants were estimated by quantitative real-time reverse transcription PCR (qRT-PCR) using SYBR Green I (Tavva et al., 2006a). We have screened at least 3 independent lines each of the *Arabidopsis* and tobacco transgenic plants expressing the *AtZFP11* gene under the control of the CfEcR+LmRXR two-hybrid switch. Though, we have observed *AtZFP11* induction levels in a wide range of methoxyfenozide concentrations, we only present data from selected methoxyfenozide doses (0, 400, 2,000, and 10,000 nM for *Arabidopsis* and 0, 16, 400, 2,000, and 10,000 nM for tobacco). Total RNA was isolated from 100 mg of tobacco and *Arabidopsis* seedlings using 1 mL of TRIzol® reagent (Invitrogen™, Life Technologies, Carlsbad, CA). The total RNA isolated using TRIzol reagent was purified by running the samples through the Qiagen columns (RNeasy Plant Mini Kit, Qiagen Inc., Valencia, CA) com-

bined with an on-column DNase digestion (RNase-Free DNase set, Qiagen Inc.) to insure DNA-free RNA preparations. First-strand cDNA synthesis was performed by using the StrataScript™ First Strand synthesis system (Stratagene, Cedar Creek, TX). DNase-treated RNA samples were tested for genomic DNA contamination by using the minus reverse transcriptase (–RT) controls in parallel to qRT-PCR reactions.

Real-time PCR quantification of the *AtZFP11* gene transcript was performed by designing specific oligonucleotide primers using PrimerQuestSM software (Integrated DNA Technologies, Coralville, IA) to amplify a 165-bp fragment (for: 5' TCC CAT GGC CTC CCA AGA ATT ACA 3'; Rev: 5' GGT TTG CAA TAG GTG TGT GGT GGT 3'). Polymerase chain reactions were carried out in an iCycler™ iQ detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I to monitor dsDNA synthesis. Serial dilutions (10^{-3} to 10^{+2} pg/μl) of the control plasmid (*AtZFP11* gene cloned in the pGEM®-T Easy vector) were used as an external control to generate a standard curve. For negative controls, the cDNA samples of wild-type untransformed tobacco and Arabidopsis and DNase-treated minus RT controls were used. Real-time PCR amplification was performed in a total volume of 20 μl reaction mixture containing 1 μl of cDNA or control plasmid, gene-specific primers, SYBR® Green I (Molecular Probes, Eugene, OR) and Platinum® *Taq* DNA polymerase (Invitrogen™, Life Technologies, Carlsbad, CA). Each sample was loaded in triplicate and the experiments were repeated twice using the following thermal cycling program conditions: initial denaturation for 2 min at 95°C, 95°C 30 s, 55°C 30 s, and 72°C 30 s for 35 cycles followed by 5-min extension at 72°C.

Melt curve analysis (Ririe et al., 1997) was done to characterize the gene-specific dsDNA product by slowly raising the temperature (0.2°C/10 s) from 60°C to 95°C with fluorescence data collected at 0.2°C intervals. The starting amount of the *AtZFP11* gene transcript in each sample was calculated using standard curve (logarithm of the starting quantity versus threshold cycle) generated for *AtZFP11*–pGEM-T Easy plasmid dilutions by the iCycler™ iQ Optical System Software (Bio-Rad Laboratories, Hercules, CA).

In order to compare the *AtZFP11* transcript level from different transgenic plants, the average starting quantity of the *AtZFP11* gene was normalized to the average starting quantity of the α -tubulin gene, which is assumed to be at a constant level in all the samples. The Arabidopsis (for: AAG GCT TAC CAC GAG CAG CTA TCA and Rev: ACA GGC CAT GTA CTT TCC GTG TCT) and tobacco (for: ATG AGA GAG TGC ATA TCG AT and Rev: TTC ACT GAA GGT GTT GAA) α -tubulin-specific primers amplified a 108- and a 240-bp fragment, respectively.

RESULTS

Stable Transformation of Arabidopsis and Tobacco Plants Using the 35S:*AtZFP11* Construct

The expression levels of the *AtZFP11* gene in wild-type control Arabidopsis plants are extremely low and no mutant phenotype is presently associated with this gene. To determine the effect of over-expressing *AtZFP11* on plant growth and development, the *AtZFP11* gene was cloned under the control of a constitutive 35S promoter (Fig. 1b). We had difficulty in recovering both Arabidopsis and tobacco transgenic plants constitutively expressing the *AtZFP11* gene (Fig. 2). Approximately 60 transgenic Arabidopsis plants containing the 35S:*AtZFP11* construct were recovered from two independent transformation experiments. Most of the lines were severely dwarfed with deformed leaves and did not set seed. We were able to collect seed from a few of these lines, of which only seed from two lines was germinated on kanamycin containing media (Fig. 2a,b). All the 35S:*AtZFP11* tobacco plants regenerated displayed an altered phenotype while growing on kanamycin containing media and continued to display abnormal phenotypes after transfer into soil (Dinkins et al., 2003). Leaves were smaller and curled, roots were more numerous, thicker, and more branched when compared to normal wild-type tobacco plants. Most of the transgenic tobacco plants did not produce viable seed and those that did failed to germinate on kanamycin media suggesting that these plants did not contain T-DNA (Fig. 2c). However, seed collected from one tobacco and two Arabidopsis

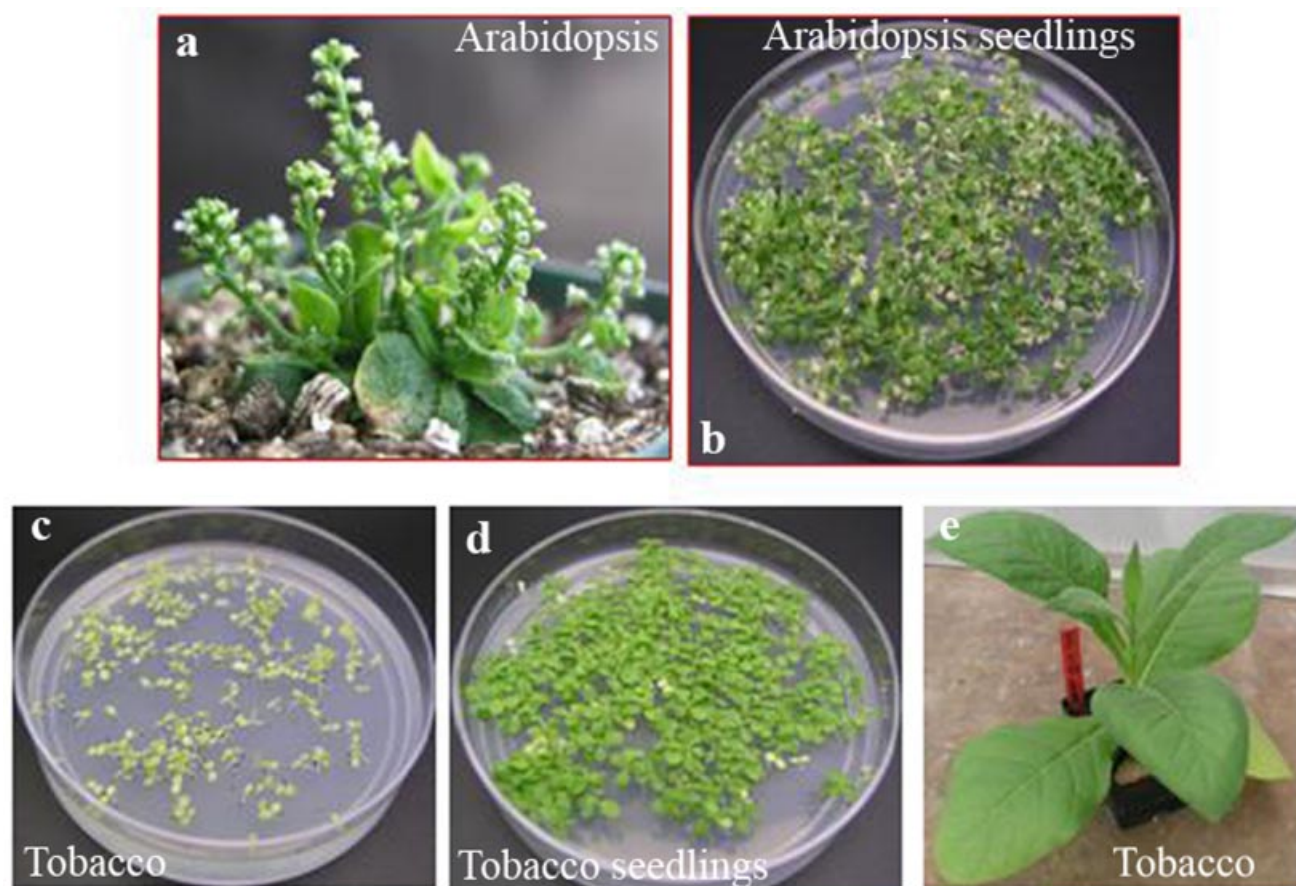


Fig. 2. Phenotype of tobacco and Arabidopsis plants expressing the *AtZFP11* gene under a constitutive 35S promoter. **a:** Transgenic Arabidopsis plants overexpressing the *AtZFP11* gene under the control of a 35S promoter. **b:** T_2 seed collected from the T_1 Arabidopsis lines were germinated on agar media containing 50 mg/L kanamycin. **c:**

T_1 seed collected from the T_0 tobacco line did not survive on kanamycin containing media. **d:** T_1 seed collected from low-expressing T_0 tobacco line survived on kanamycin containing media. **e:** A low-expressing tobacco line growing normally in the greenhouse.

transgenic lines was able to germinate on kanamycin containing media. The seedlings from this tobacco line looked normal and the plants grew well in the greenhouse (Fig. 2d,e). The seedlings from the two Arabidopsis lines looked abnormal and showed retarded growth (Fig. 2a,b).

Stable Transformation of Arabidopsis and Tobacco Plants With the *AtZFP11* Gene Placed Under the Control of CfEcR+LmRXR Two-Hybrid Gene Switch

We cloned the *AtZFP11* gene under the control of CfEcR+LmRXR two-hybrid gene switch (Fig. 1a) to determine whether the controlled expression of

the *AtZFP11* gene would produce phenotypically normal plants. Several transgenic Arabidopsis and tobacco lines were generated and the primary transformants were transferred to the greenhouse (Figs. 3–7). Less than 20% of the transgenic lines displayed an abnormal phenotype in the absence of applied methoxyfenozide, and the majority of the transformants grew well in the greenhouse (Figs. 4 and 7). Irrespective of the phenotypic abnormalities observed, all the Arabidopsis and tobacco lines produced viable seed. However, the number of seed collected from the lines that showed the abnormal phenotype was less compared to the amount of seed collected from the

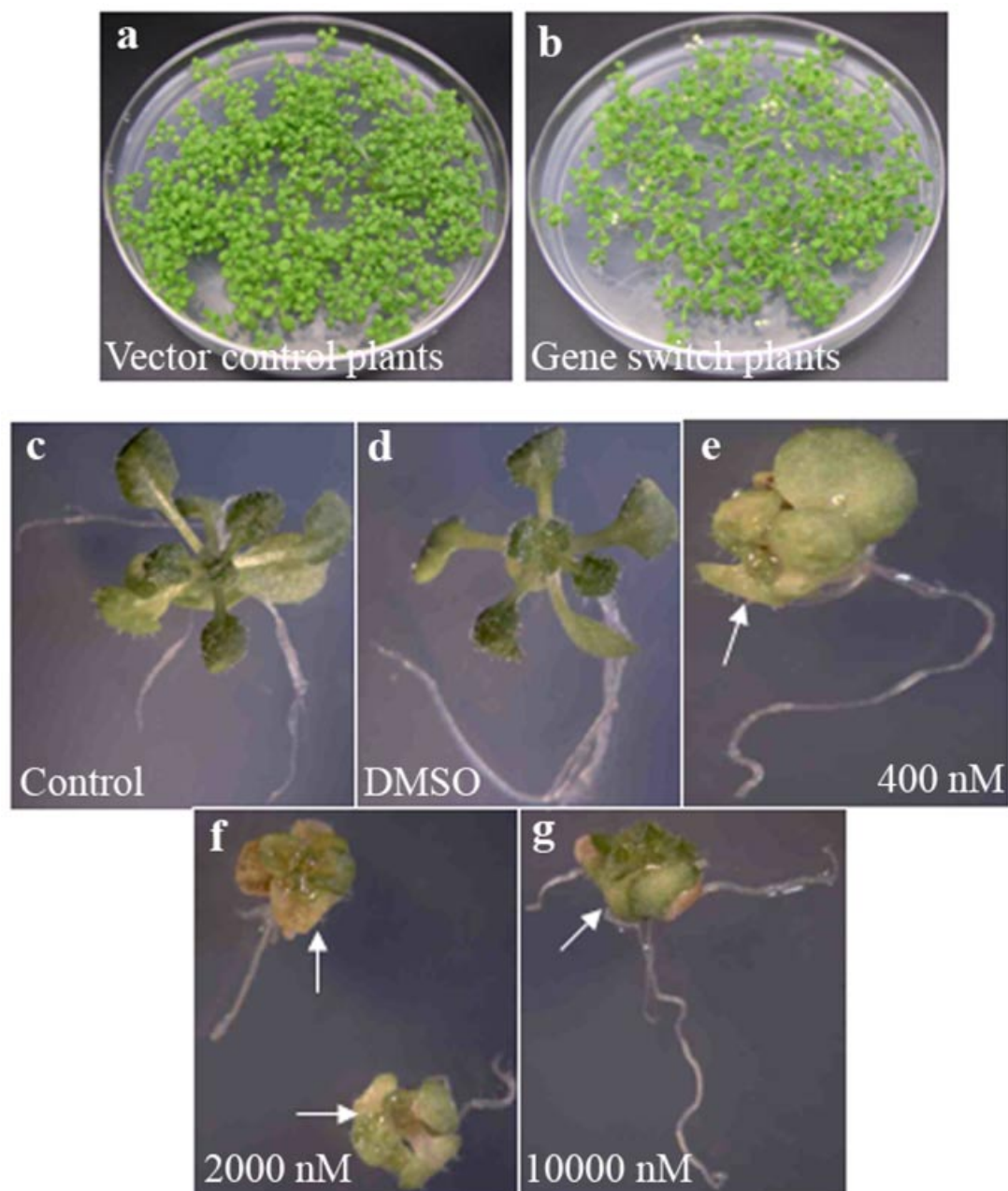


Fig. 3. Methoxyfenozide-inducible *AtZFP11* phenotype in Arabidopsis seedlings. Pictures were taken 20 days after plating the seed on agar media containing different methoxyfenozide concentrations. **a:** Vector control T2 Arabidopsis seedlings growing on kanamycin containing media. **b:** Seed collected from the T2 transgenic Arabidopsis plant developed for the p2300CfE:LmR:AtZFP11 construct were plated

on the agar media containing 50 mg/L kanamycin and DMSO. **c–g:** Microscopic pictures of the T2 transgenic Arabidopsis seedlings. **c:** Vector control; **d–g:** Gene switch seedlings collected from different methoxyfenozide treatments showing abnormal phenotype (→). **(d)** 0 (DMSO), **(e)** 400, **(f)** 2,000, and **(g)** 10,000 nM.

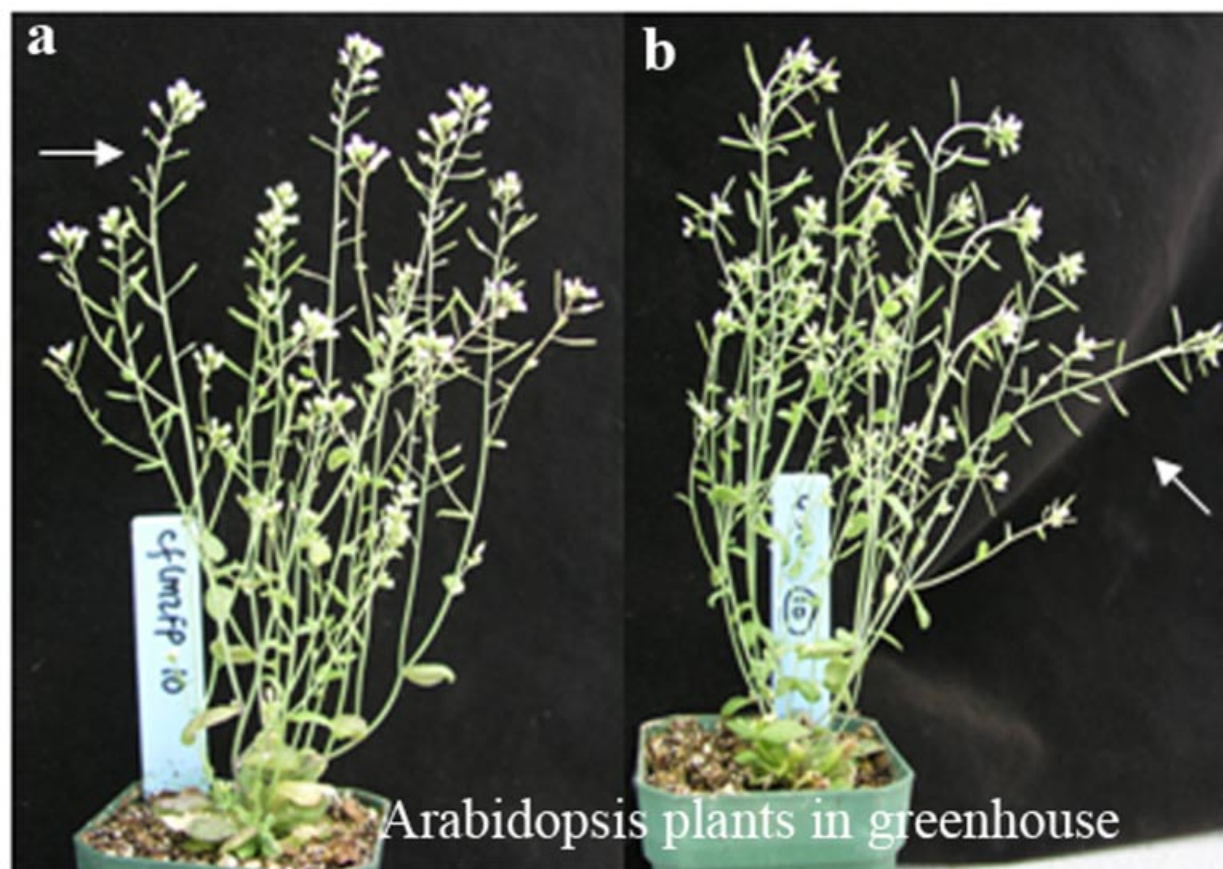


Fig. 4. Transgenic Arabidopsis plants growing in the greenhouse. Transgenic Arabidopsis plants carrying the *AtZFP11* gene under the control of the CfEcR+LmRXR

switch were transferred to the greenhouse from the DMSO (a) and 400 nM methoxyfenozide (b) treatments.

transgenic plants that were phenotypically normal (Fig. 7c–e).

Methoxyfenozide Induces the *AtZFP11* Phenotype

Seed collected from the Arabidopsis and tobacco transgenic lines was screened for the methoxyfenozide-inducible phenotype. T₂ Arabidopsis and the T₁ tobacco seed were plated on agar media supplemented with kanamycin and different doses of methoxyfenozide (0, 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 nM). We observed dose-dependent *AtZFP11*-induced phenotype both in Arabidopsis and tobacco transgenic plants (Figs. 3 and 5). The transgenic plants displayed an altered phenotype as early as the tissue culture medium stage with the application of 16 nM of methoxyfenozide

in tobacco plants and 80 nM methoxyfenozide in Arabidopsis plants. Leaves were greenish, crinkled, and swollen, roots were very thick and rigid compared to wild-type plants (Figs. 3 and 5). The *AtZFP11*-induced phenotype was very conspicuous at higher doses of methoxyfenozide and no such phenotypes were observed in either tobacco or Arabidopsis seedlings grown without methoxyfenozide (Figs. 3d, 5a, and 6a).

qRT-PCR Analysis of Methoxyfenozide-Inducible *AtZFP11* Expression Level

The qRT-PCR analysis showed that the expression levels of the *AtZFP11* gene in wild-type control Arabidopsis plants (Col ER) were extremely low (5.29×10^3 copies of *AtZFP11*/μg of total RNA).

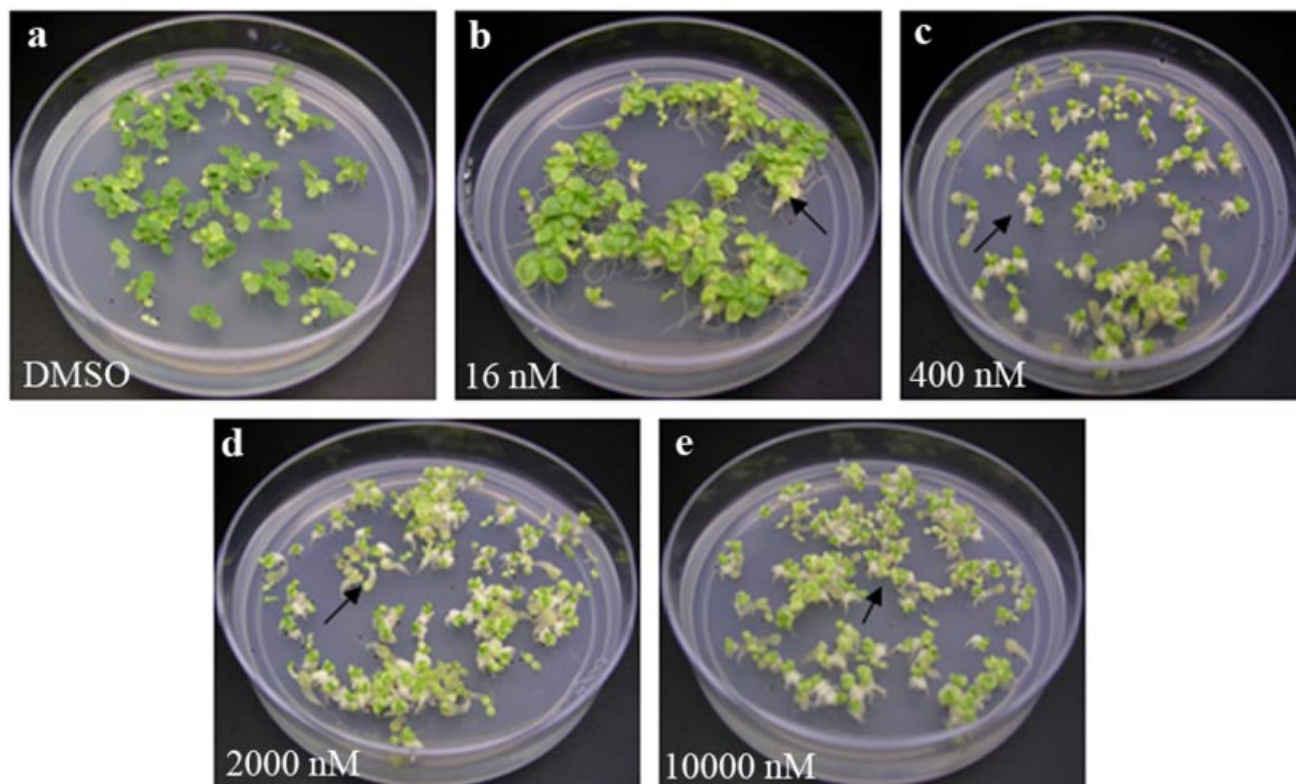


Fig. 5. Methoxyfenozide-inducible *AtZFP11* phenotype. Transgenic tobacco seedlings expressing the *AtZFP11* gene under the control of the CfECR+LmRXR two-hybrid gene switch and methoxyfenozide. Seed collected from the T₀ transgenic tobacco plant developed for the p2300CfE:LmR:*AtZFP11* construct were plated on agar

media containing 300 mg/L kanamycin and different concentrations of methoxyfenozide. Pictures were taken one month after plating the seed on different methoxyfenozide concentrations and the phenotypic abnormalities were marked (→) on photographs. (a) 0 (DMSO), (b) 16, (c) 400, (d) 2,000, and (e) 10,000 nM.

In 35S:*AtZFP11* *Arabidopsis* plants, the average *AtZFP11* mRNA levels increased to 2.24×10^5 copies/ μ g of total RNA, which is 48-fold higher than the *AtZFP11* mRNA levels observed in the wild-type control plants (Fig. 8a). In *Arabidopsis* plants where *AtZFP11* was under the control of the gene switch, DMSO treatment showed 1.1×10^5 copies of *AtZFP11*/ μ g of total RNA and the levels were increased to 6.0×10^5 copies/ μ g of total RNA with the application of 400 nM methoxyfenozide and reached a maximum of 1.63×10^6 copies/ μ g of total RNA with the application of 10,000 nM methoxyfenozide. The *AtZFP11* mRNA levels observed in 400- and 10,000-nM methoxyfenozide-treated *Arabidopsis* plants was 5.4- and 14.74-fold higher, respectively, than the *AtZFP11* levels observed in the DMSO treated plants. In transgenic plants where

AtZFP11 was placed under the control of the gene switch, the *AtZFP11* mRNA levels recorded in the 10,000-nM methoxyfenozide-treated *Arabidopsis* plants were 7.2- and 307.4-fold higher, respectively, than the mRNA levels observed in 35S:*AtZFP11* and wild-type *Arabidopsis* plants.

Most of the T₀ transgenic tobacco plants devel-

Fig. 6. Transgenic tobacco seedlings showing reduction of the induced phenotype upon withdrawal of the ligand. (i) Tobacco seedlings growing on different concentrations of methoxyfenozide were transferred to fresh agar media containing 300 mg/L kanamycin, without added inducer. Pictures were taken immediately after transferring onto fresh media. (ii) Tobacco seedlings at 15 days after withdrawal of ligand started exhibiting normal phenotype (→). (a) 0 (DMSO), (b) 16, (c) 400, (d) 2,000, and (e) 10,000 nM.

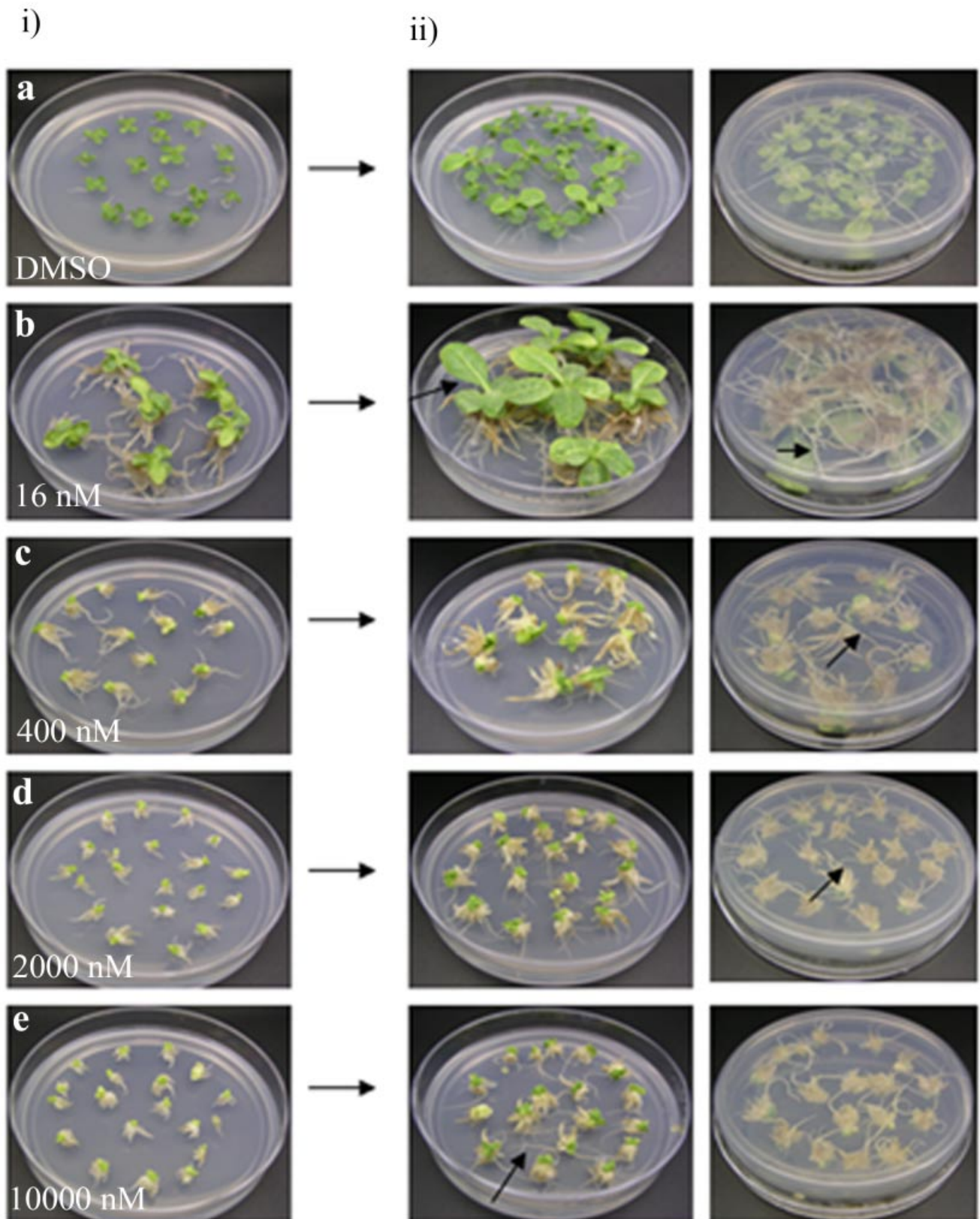


Figure 6

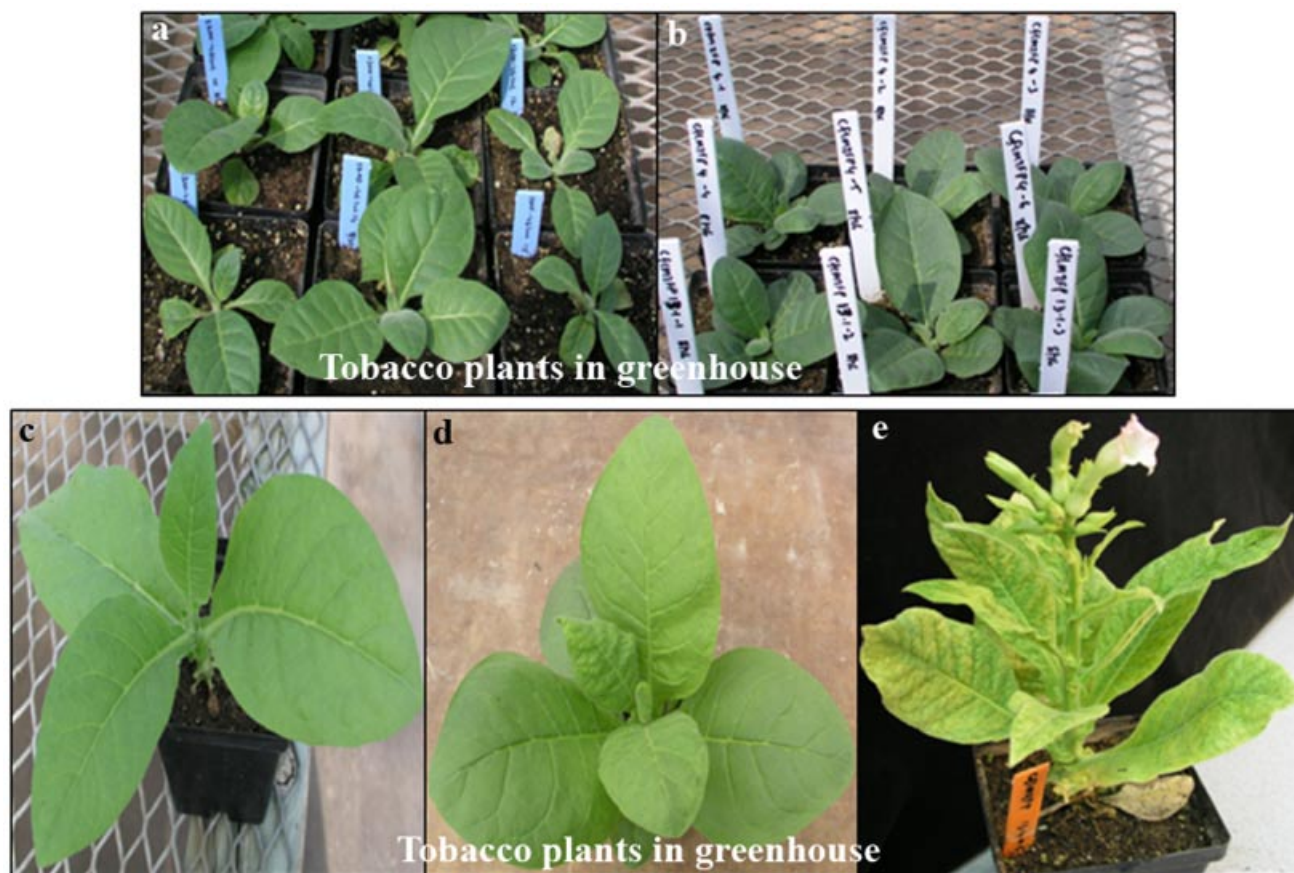


Fig. 7. Transgenic tobacco plants growing in the greenhouse. **a**: Transgenic tobacco plants carrying the vector backbone and reporter expression cassette (vector control plants). **b–d**: Transgenic tobacco plants carrying the

AtZFP11 gene under the control of the CfEcR+LmRXR switch. **e**: Transgenic tobacco plant carrying the *AtZFP11* gene under the control of the CfEcR+LmRXR switch exhibiting an abnormal phenotype.

oped for the 35S:*AtZFP11* construct did not set seed and only one line did set seed and germinated normally and showed low levels of *AtZFP11* expression (1.06×10^5 copies/ μg of total RNA), as verified by qRT-PCR. No abnormal phenotype was associated with this line (Fig. 2d,e). qRT-PCR data on the tobacco plants carrying the gene switch revealed that the *AtZFP11* expression reached maximum (4.4×10^6 copies/ μg of total RNA) with the application of 16 nM of methoxyfenozide. The *AtZFP11* mRNA levels were increased by 13.26-, 11.19-, 5.31-, and 7.78-fold, respectively, in 16-, 400-, 2,000-, and 10,000-nM methoxyfenozide-treated tobacco plants when compared to the *AtZFP11* mRNA levels observed in DMSO-treated plants. The *AtZFP11* mRNA levels observed in 16-nM-treated tobacco plants were 41.97-fold higher than the *AtZFP11* levels ob-

served in the tobacco plants expressing *AtZFP11* under the control of the 35S promoter (Fig. 8b).

Recovery of Normal Phenotype

To determine whether or not the induced plants can recover from the induced phenotype, tobacco seedlings that were growing on inducing media for one month were transferred to fresh agar media without any methoxyfenozide. When maintained on agar plates without methoxyfenozide, tobacco plants, at least the seedlings that were transferred from the plates containing lower doses of methoxyfenozide, started looking like normal plants (Fig. 6b). Plants originating from the higher doses (400, 2,000, and 10,000 nM) did not recover (Fig. 6c–e), although they began to appear “less abnormal”

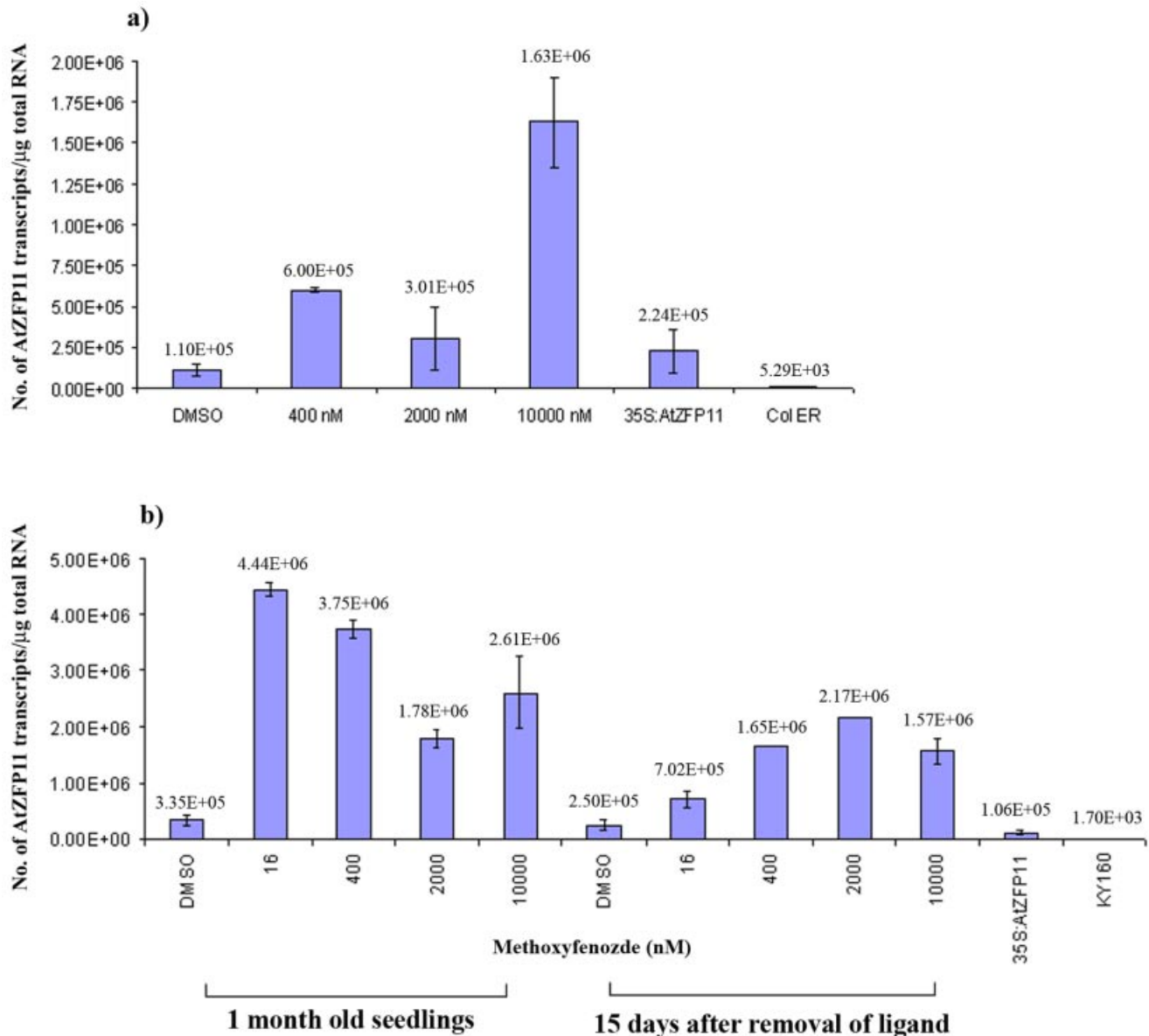


Fig. 8. Expression of the *AtZFP11* gene in transgenic tobacco and Arabidopsis plants. The values in the histogram represent the *AtZFP11* expression levels adjusted to α -tubulin expression across all samples. Units are given as number of *AtZFP11* molecules/ μ g of total RNA. Data represent an average of 3 replicates \pm SD. **a:** Graph showing *AtZFP11* expression levels in Arabidopsis seedlings grown for 20 days on DMSO, 400 nM, 2 μ M, and 10 μ M concentrations of methoxyfenozide. This graph also shows *AtZFP11* expression levels in 35S:*AtZFP11* Arabidopsis

plants and wild-type control plants (Col ER). **b:** Graph showing *AtZFP11* expression levels in tobacco seedlings grown for one month on DMSO, 16, 400, 2,000, and 10,000 nM concentrations of methoxyfenozide and 15 days after removal of the ligand. This graph also shows *AtZFP11* expression levels in transgenic tobacco developed for the construct where the *AtZFP11* gene was cloned under the control of a 35S promoter (35S:*AtZFP11*) and wild-type control plants (KY160).

after one month following removal of the methoxyfenozide (data not shown). The *AtZFP11* expression levels went down after the EcR switch reverted to the un-induced state (Fig. 8b). The qRT-PCR data confirmed the reduction in *AtZFP11* expression levels upon withdrawal of the ligand and within 15 days the mRNA levels went down by 6.3-, 2.3-, 0.8-, and 1.7-fold, respectively, in the seedlings that were transferred from 16-, 400-, 2,000-, and 10,000-nM methoxyfenozide treatments to media containing no methoxyfenozide (Fig. 8b).

DISCUSSION

The use of molecular genetic approaches to investigate a broad range of biological pathways is not easily applied to genes that control fundamental processes of plant growth, differentiation, and reproduction. This is because manipulating such critical genes can be severely detrimental to plant growth and survival and thus precludes the generation and propagation of useful transgenic plants (Chen et al., 2003). Constitutive promoters are not suitable to regulate such critical developmental genes. Therefore, the ability to tightly regulate gene expression in plant cells will be an effective tool for the elucidation of gene function. Inducible gene regulation systems based on specific receptor and chemical inducers are useful for investigating gene function. A number of inducible gene regulation systems have been developed to regulate the expression of transgenes in plants (Zuo and Chua, 2000; Padidam, 2003; Tang et al., 2004). The EcR-based gene switch is one of the most applicable gene regulation systems available since the chemical ligand required for its regulation is already registered for field use (Dhadialla et al. 1998; Palli et al. 2005a).

EcR gene switches also have possible applications in biotechnology that are not provided by constitutive gene expression. Unger et al. (2002) developed an EcR switch comprised of the *Ostrinia nubilalis* EcR (LBD), maize C1 activation domain, and GAL4 DNA binding domain to induce the expression of the fertility-restorer gene MS45 in male

sterile ms45 maize mutants. Methoxyfenozide applied to the soil (1,200 mg/pot) induced the MS45 gene to sufficiently high expression levels and for a time required to effect self-pollination. The VGE system developed by Padidam et al. (2003) was used to conditionally express tobacco mosaic virus (TMV) coat protein gene expression. Arabidopsis plants containing the inducible TMV coat protein under ecdysone receptor switch control exhibited resistance to virus infection upon addition of the ligand (Koo et al., 2004). Although all these studies are based on the ecdysone receptor gene regulation system, they require large quantities of chemical ligand for the induction of target gene expression. The experiments reported earlier (Palli et al. 2003, 2005b; Tavva et al. 2006b) demonstrated that the use of LmRXR as a partner with CfEcR improved the sensitivity of the CfEcR two-hybrid switch (CfEcR+LmRXR) from micromolar to nanomolar concentrations of chemical ligand and should provide a powerful tool in biotechnology and functional genomic studies.

In animal systems, the zinc finger protein gene family is one of the largest groups of transcription factors and its members play a wide and important role in growth and development. Similarly, plants also contain a large array of zinc finger proteins, with many being unique to plants (Takatsui, 1999). Only four of the 30 Arabidopsis single C2H2 zinc finger proteins have a known function based on mutant phenotypes, which is probably due to functional redundancy. Ectopic expression, using the gain-of-function strategy, is particularly attractive when no mutant phenotype exists for the gene of interest, as is the case for *AtZFP11*. Overexpression of the *AtZFP11* gene in Arabidopsis under a constitutive promoter resulted in severely deformed and dwarfed plants that did not survive outside the culture medium (Fig. 2). Overexpression of *AtZFP11* in tobacco also resulted in severely reduced stem elongation, abnormal leaf shape, and sterility (Dinkins et al., 2003).

Both Arabidopsis and tobacco transgenic plants developed for the p2300CfE:LmR:AtZFP11 construct exhibited the methoxyfenozide-inducible

AtZFP11 phenotype. The induced phenotype observed in these plants is similar to what we observed with 35S:*AtZFP11*-expressing plants (Figs. 2, 3, and 5), confirming that controlled expression of *AtZFP11* is necessary to recover healthy transgenic plants. No phenotype was associated with either *Arabidopsis* or tobacco transgenic seedlings in the absence of ligand. Several plants germinated on methoxyfenozide free-medium were transferred to the greenhouse and their development and growth in the greenhouse were monitored over the entire life cycles (Figs. 4 and 7). We have screened transgenic *Arabidopsis* plants for three generations and tobacco for two generations, and all these lines continued to display a methoxyfenozide-inducible *AtZFP11* phenotype, indicating that the CfEcR+LmRXR switch is stable in subsequent generations and able to trigger *AtZFP11* expression and the related phenotype with the application of methoxyfenozide. To verify that the transgenic plants were able to induce *AtZFP11* expression with the application of methoxyfenozide, qRT-PCR was carried out and demonstrated that the *AtZFP11* gene is expressed when placed under the control of the CfEcR+LmRXR switch (Fig. 8). Moreover, in tobacco seedlings *AtZFP11* transcript levels went down upon removal of the ligand and began to revert to normal phenotype (Figs. 6 and 8b).

By using the EcR gene switch, we were able to regulate the expression of a critical gene, *AtZFP11*, in both *Arabidopsis* and tobacco transgenic plants. The CfEcR+LmRXR two-hybrid switch is an ideal system in functional genomics studies as it required a non-toxic inducer, methoxyfenozide; uses a very low (nM) concentration of ligand; and, the transgenic plants exhibited recovery of the normal phenotype upon withdrawal of the ligand. The transgenic plants expressing the *AtZFP11* gene under the control of the CfEcR+LmRXR switch generated in the present study should be a valuable material in functional genomic applications to discover the downstream target genes of the *AtZFP11* transcription factor in plants, which is otherwise difficult when the *AtZFP11* gene is over-expressed under the control of a constitutive promoter due to the problems associated in obtaining seed.

ACKNOWLEDGMENTS

We thank Kay McAllister, Jeanne Prather, Travis Timberlake, and Ray Stevens for technical and greenhouse help. We also thank Dr. Indu Maiti, University of Kentucky, for providing the plasmids containing the MMV and FMV promoters. This research was supported in part by funds provided by the Kentucky Tobacco Research and Development Center (KTRDC), University of Kentucky. This report (No. 06-08-114) is published with the approval of the Director of the Kentucky Agricultural Experiment Station.



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